Identifying serum exosome microRNA signatures in melanoma patients

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Abstract

Melanoma is the 6th most common cancer in the U.S. with a median survival rate of less than one year. Early diagnosis of melanoma is challenging because the traditional histopathologic methods cannot sufficiently distinguish benign from malignant lesions. Minimally invasive and dependable determinants that can refine the individualized diagnosis are needed. Exosomes are small membrane vesicles (~30-120nm) that are secreted from cells and contain protein and RNAs. They are implicated in cell-cell communication. Previous studies have shown that miRNAs in tumor exosomes contribute to progression of disease via mRNA silencing. Characterizing the differences in expression of exosomal miRNA between melanoma and non-melanoma patients could lead to a method for earlier and more quantitative diagnosis. In the current study, blood exosomes were isolated from non-melanoma subjects and Stage I melanoma patients. miRNA array was applied to compare the miRNA expression profiles between non-melanoma controls and Stage I melanomas. Real time RT-PCR was performed to confirm some of the differentially expressed miRNAs in these two groups. We showed that a panel of exosomal miRNAs (such as hsa-miR-1228 and hsa-miR-1825) has significant changes in Stage I melanoma patients vs non-melanoma controls. These results provide a starting point for further characterization of exosomal miRNA signatures in melanoma patients. This research could lead to a minimally invasive method for specific diagnosis of malignant melanoma.

Introduction

- **Exosomes are 30-120nm vesicles secreted by cells that contain RNA and protein implicated in cell-cell communication and commonly found in body fluids such as blood and urine.**
- **microRNA (miRNA) is small, noncoding RNA that posttranscriptionally silences mRNA.**
- **miRNA in tumor exosomes have been shown to contribute to malignant progression**
- **Analyzing exosomal miRNA signatures in melanoma patients could identify a potential biomarkers for diagnosis & prognosis**

Methods

Patient Groups:

- Non-melanoma
- Stage I Melanoma

1. Exosomes were isolated from serum using the ExoQuick protocol.
2. RNA was isolated from exosomes using a modified mirVARNA RNA isolation protocol.
3. miRNA array was conducted using Affymetrix miRNA array 2.0.
4. Differentially expressed miRNAs between the two sample groups were analyzed by ANOVA.
5. RT-PCR was performed to validate differentially expressed miRNAs identified in microarray screen.

Results

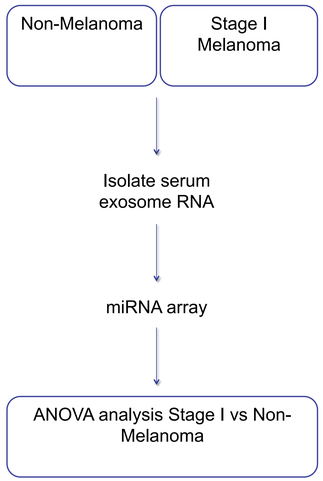


Figure 1. Initial microarray screen.

miRNAs identified in microarray screen:

miRNA	Fold change	P-Value
miR-23a	1153.14	7.902E-10
miR-26a	1069.13	3.20E-09
miR-191	794.92	7.71E-10
let7a	711.23	1.65E-07
miR-221	601.23	1.43E-08

Table 1. Selected upregulated miRNAs (stage I vs. non-melanoma) (*P*<0.05, -2>FC>2)

miRNA	Fold change	P-Value
miR-940	-122.15	5.03E-07
miR-1825	-104.56	7.81E-06
miR-1228	-94.67	6.87E-06
miR-4258	-84.13	6.91E-06
miR-1281	-62.27	4.61E-05

Table 2. Selected downregulated miRNAs (stage I vs. non-melanoma) (*P*<0.05, -2>FC>2)

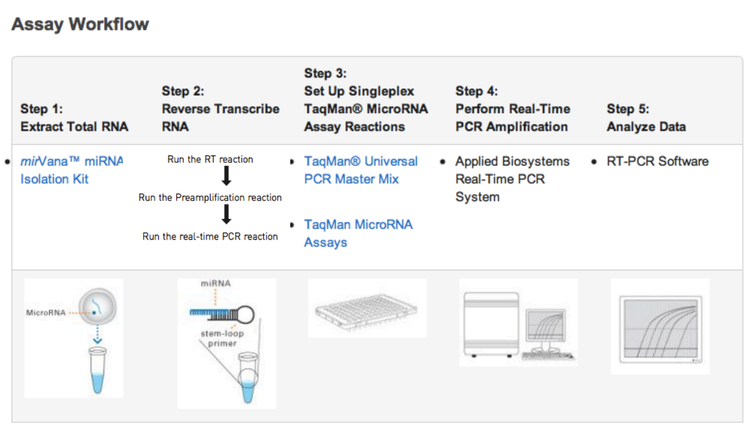
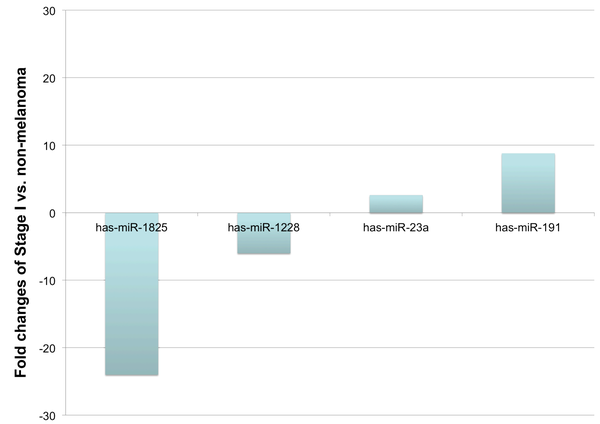


Figure 2. RT-PCR workflow.



Graph 1. Validation of differentially expressed miRNAs (4 miRNAs confirmed microarray results).

Patient Group	Total N	Sex (M/F)	Age Range	Mean Age
Non-melanoma	10	4/6	28-81	52
Stage I	15	9/6	23-81	53

Table 3. Patient demographics.

Conclusions

- **There are specific exosomal miRNAs found in Stage I melanoma patients compared to non-melanoma patients.**
- **Exosomal miRNAs hsa-miR-122, hsa-miR-1825, hsa-miR-23a & has-miR-191 have significant changes in Stage I melanoma patients vs non-melanoma controls.**
- **These results provide a starting point for further characterization of exosomal miRNA signatures in melanoma patients.**
- **This research could lead to a minimally invasive method for specific and early diagnosis of malignant melanoma.**

Acknowledgements

Research supported by NCI R25 grant University of Louisville Cancer Education Program NIH/NCI (R25-CA134283), grant from Melanoma Research Foundation, and University of Louisville Clinical & Translational Science Pilot Grant Innovative Award to K.M.M.

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Tobacco-Induced Dysregulation of Matrix Metalloproteinases in HL-60 cells.

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Introduction

- Matrix metalloproteinases (MMPs) are a family of approximately 28 secreted and membrane-bound zinc-endopeptidases involved in angiogenesis and tissue remodeling, the two key determinants of cancer growth.
- There are strong correlations between smoking prevalence and cancers such as lung, oral and blood cell cancers as well as multiple infectious diseases.
- Nicotine (3-(1-methyl-2-pyrrolidinyl) pyridine), a key toxic component of tobacco, is thought to dysregulate MMP secretion in innate immune cells in an α_7 nicotinic acetylcholine receptor (nAChR)-dependent manner.
- HL-60 cells were derived from an individual with acute promyelocytic leukemia and are commonly employed as model of innate immune cell differentiation and function.
- IL-8 is a leukocyte chemoattractant which is a growth factor for malignant melanoma and regulates angiogenesis during cancer progression.
- **We examine the influence of nicotine on MMP's and IL-8 production in monocytic cells.**

Materials and Methods

- HL-60 cells were cultured in RPMI 1640 medium along with 10% FBS and DMSO at 37° C in an atmosphere of 5% CO_2 and 100% humidity.
- HL-60 cells were differentiated into monocytes by adding 16 nM PMA for 72 hours followed by nicotine treatment as indicated in the figures.
- MMP2 and MMP9 activity were determined by 7% gelatin gel zymogram
- Culture supernatants were collected from nicotine treated HL60 cells at different time interval and MMP9 and IL-8 concentration were determined by ELISA.
- Statistical significance between groups was evaluated by ANOVA using the GraphPad prism Software. Differences between groups were considered significant at the level of $p < 0.05$.

Results

***Figure* 1: MMP2 relative quantification from zymogram.**

Nicotine does not influence MMP2 release from monocytic cells .

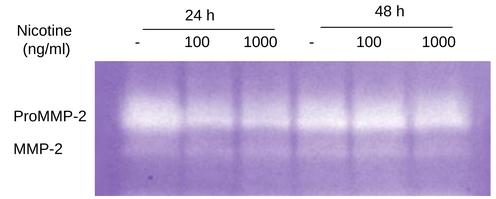
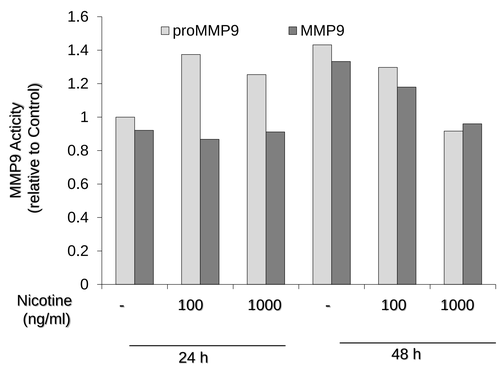


***Figure* 2: Nicotine suppresses MMP9 release from monocytic cells**
A; zymogram/densitometry. B; ELISA



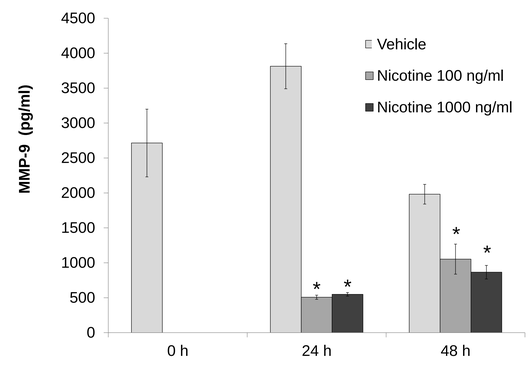
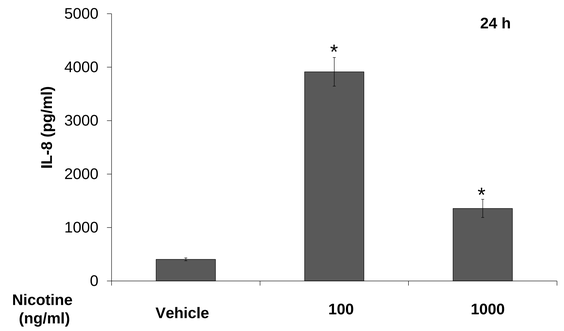


***Figure* 3: IL-8 ELISA. 100 ng/ml nicotine leads to significant increase in IL-8 production.**
***p < 0.05, when compared to vehicle.**



Conclusions

- Nicotine decreased the MMP9 activity in a dose- and time-related manner.
- Nicotine decreased the MMP2 production at 48 h but had no significant effect on the MMP2 activity in monocytic HL60 cells.
- Nicotine treatment leads to increased IL-8 production which correlates with increased MMP-9 activity at 24 h
- Future studies will assess the importance of dysregulated MMP and IL-8 production in HL-60 chemotaxis studies.

Acknowledgements

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NIDR R01DEO19826 (DAS)

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Development of a Blood Test as an Innovative Screening Tool for Lung Cancer

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Introduction

Lung cancer is the major cause of cancer-related deaths worldwide, accounting for about 1.3 million deaths annually. Approximately $10.3 billion is spent on lung cancer treatment in the United States each year. Often, clinical symptoms only appear during later stages of cancer development at which point the cancer may have metastasized. Various screening strategies have been tested for detection of early stage lung cancer but only one cumbersome technique (low-dose computed tomography) has shown minor promise. We have developed an inexpensive and fast alternative blood test that will detect antibodies appearing early in the development of lung cancer. The test involves flow cytometric analyses of A549 (human lung adenocarcinoma) cells, as well as other cancer cell lines, incubated with dilute patient serum and a secondary anti-human IgG or IgM antibody (tagged with the fluorescent tag, R-Phycoerythrin or Allophycocyanin respectively). Several groups have examined the serum titer of antibodies against specific lung cancer antigens as a possible screening tool, but the strength of our approach is that it is a broad-spectrum screen that will identify antibodies against a variety of known and unknown cell surface antigens. In addition to detecting lung cancer in its earliest stages, preliminary data also suggest our technique offers the exciting possibility of identifying novel human lung cancer antigens that may serve as targets for future immunotherapy. Supported by grant R25-CA-134283 from the National Cancer Institute.

Materials & Methods

Flow Cytometry - Extracellular Antigen Detection

1) Suspend 1x10^5 human cancer cells, or immortalized normal human cells, in 100 ul staining buffer (PBS + 1% Fetal Bovine Serum). Add human serum to a final dilution of 1:50 and incubate on ice for 30 minutes.
2) Wash cells twice at 1500 RPM for 5 minutes with 2 ml of staining buffer (PBS with 1% FBS).
3) Add anti-human IgG, F_C fragment specific (conjugated with PE) and anti-human IgM, Fc fragment specific (conjugated with Allophycocyanin) to a final dilution of 1:100 and incubate on ice for 30 minutes in the dark.
4) Wash cells twice with 2 ml of staining buffer.
5) Resuspend cells in 500 ul of staining buffer and test for antibody binding via flow cytometry.

Western Blot Procedure

1) 30 ug of cell lysate was mixed with 2x Laemmli sample buffer (containing BME) in a 1:1 dilution. Total volume was normalized between samples by additional sample buffer. Samples were heated at 100°C for 10 minutes and then cooled to room temperature. Samples were run at 100 volts on a 4-20% Mini-PROTEAN TGX precast polyacrylamide gel for one hour at 25°C.
2) Protein was transferred to activated PVDF membrane at 4°C for 1 hour at 100 volts.
3) Endogenous peroxidase activity was then inactivated by incubating the membrane in 3% H_2O_2 for 15 minutes.
4) Membranes were washed with TBST (0.1% Tween-20). 1x for 5 minutes to remove residual H_2O_2.
5) Membranes were blocked with 5% milk solution overnight at 4°C.
6) Membranes were washed 3x for 5 minutes and 1x for 10 minutes with TBST.
7) Membranes were incubated overnight at 4°C with serum (1:100 dilution in 3% milk)
8) Membranes were washed 3x for 5 minutes and 1x for 10 minutes with TBST.
9) Membranes were blocked with 5% goat serum for 72 hrs, followed by washes as previously described.
10) Membranes were incubated for 45 min at 4°C with goat anti-human IgG (F_C fragment specific, HRP conjugated) and then washed as previously described.

Statistical Analysis

1) Data were analyzed using Microsoft Excel 2010. P-values were calculated from a two-way student's t-test, assuming equal variance between all groups. All student-t test values were calculated from comparing the experimental group with the respective normal serum controls unless otherwise specified.

Results

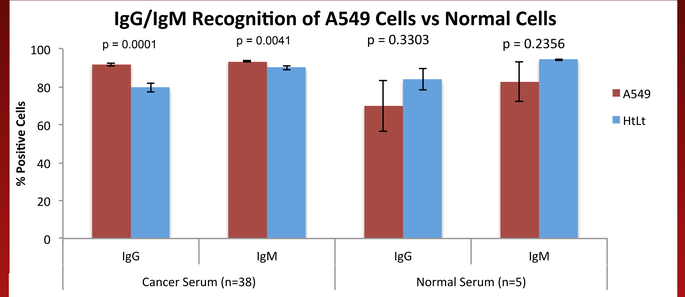


Figure 1. IgG/IgM Recognition of A549 Cells vs Normal Cells. The IgG and IgM autoantibodies in the serum from lung cancer patients (n=38) preferentially bind A549 lung adenocarcinoma cells vs immortalized human bronchial epithelial cells (HtLt) with p-values of 0.0001 and 0.0041 respectively. There was no statistically significant difference in the binding of IgG and IgM antibodies found in normal human serum (n=5).

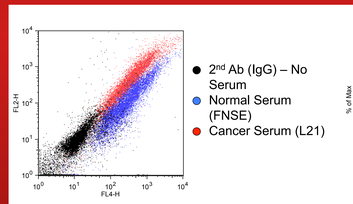


Figure 2a

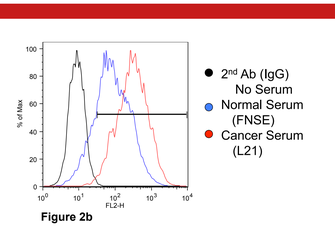


Figure 2b

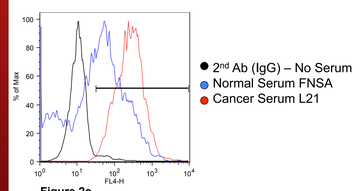


Figure 2c

Figure 2a. IgG (FL2) and IgM (FL4) shifts are visible in representative serum samples incubated with A549 cells.
Figure 2b. There is an increased amount of IgG found in lung cancer serum that binds A549 cells.
Figure 2c. There is an increased amount of IgM found in lung cancer serum that binds A549 cells.

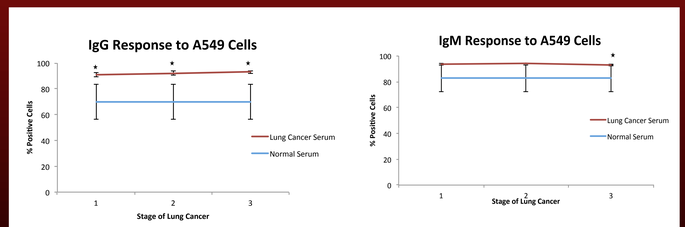


Figure 3. Stability of IgG and IgM throughout lung cancer stages 1-3. IgG recognition is significantly elevated throughout the first three stages of lung cancer, however IgM is only significantly elevated in stage 3 of lung cancer.

Table 1. P-values for IgG/IgM Response to Different Cell Lines. The autoantibody IgG and IgM response to A549 non-small lung adenocarcinoma cells were both statistically significant (p = 0.0001 and p = 0.001 respectively). Values represent the percentage of cells bound by IgG or IgM.

	IgG Response			IgM Response		
	Cancer Serum (n=39)	Normal Serum (n=6)	p-value	Cancer Serum (n=39)	Normal Serum (n=6)	p-value
H1299	71	79	0.38	91	92	0.69
A375	93	94	0.81	96	97	0.33
MCF7	71	63	0.23	89	90	0.81
A549	92	70	0.0001	93	83	0.001
DU145	23	23	0.96	98	98	0.83
Hela	77	67	0.35	98	98	0.96
HtLt	80	84	0.52	90	94	0.18
HtLt Ras	75	74	0.96	88	92	0.31

Table 2. Different measures of statistical importance indicate significant differences (p<0.05) between normal and lung cancer serum samples. In addition to the percentage of cells bound by IgG and IgM antibodies found in the serum, the geometrical mean intensity, mean intensity, median intensity, and mode of intensity were evaluated for statistical importance. These results indicate that significantly more IgG and IgM in sera from lung cancer patients bind to epitopes on the extracellular membrane of A549 cells. However, the difference between normal vs. cancer serum is largest for IgG recognition.

	IgG Response on A549 Cells			IgM Response on A549 Cells		
	Cancer Serum	Normal Serum	p-value	Cancer Serum	Normal Serum	p-value
% Positive Cells	92	70	0.0001	94	83	0.0012
Geom. Mean Intensity	164	79	0.0199	295	209	0.3529
Mean Intensity	264	155	0.0435	429	357	0.5719
Median Intensity	172	78	0.0223	304	220	0.3930
Mode of Intensity	202	107	0.0941	315	296	0.8538

Table 3. Sensitivity and specificity values for assessing serum IgG or IgM for lung cancer. With a cutoff value of 85%, the sensitivity and specificity of measuring IgG binding to A549 cells is 0.87 and 0.60 respectively. Increasing the cutoff value to 88% decreases sensitivity to 0.81, but increases the specificity to 0.80. This results in a positive likelihood ratio (+LR) of 4.05, which would be ideal for annual or semi-annual screening of large populations of patients for lung cancer.

	Cutoff Value (%)	Sensitivity	Specificity	Positive LR	Negative LR
IgG	85.0	0.87	0.60	2.17	0.22
	88.0	0.81	0.80	4.05	0.24
IgM	98.5	0.71	0.40	1.18	0.73
	98.8	0.65	0.60	1.63	0.58

Conclusions

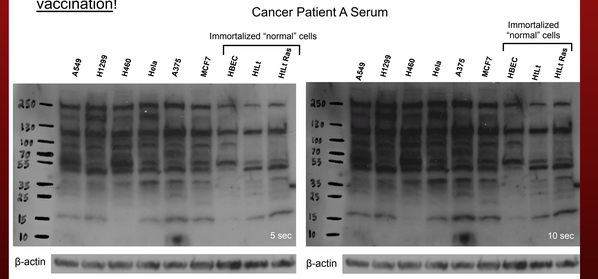
Within the past decade, several labs have begun measuring antibody titers to specific tumor autoantigens with the hope of developing a screening tool that is an effective, non-invasive, and cheap alternative to receiving low-dose CT scans annually, as recently recommended by the United States Preventative Services Task Force.

Using flow cytometric methods, we were able to show that titers of IgG and IgM specific to A549 cells were elevated in the serum of lung cancer patients (Figs. 1-2) as compared to normal human serum (p=0.0001 and p=0.001, respectively). We were also able to show that the IgG/IgM antibodies that are present in the serum of lung cancer patients bind preferentially to A549 cells vs immortalized normal bronchiolar endothelial (HtLt) cells (p<0.05), whereas the IgG/IgM antibodies found in normal human serum do not bind preferentially to A549 cells or HtLt cells (p>0.2; Fig. 3).

With the hope of being able to detect lung cancer in it's earliest stages of development, we analyzed the titer of IgG/IgM specific for A549 cells using patient sera obtained over the first three stages of lung cancer. We found that IgG levels remained elevated throughout stages 1-3 of lung cancer at a statistically significant level (p<0.03). The titer of IgM was elevated throughout the first three stages of lung cancer as well, but was only statistically significant in stage 3 (Fig. 4). Although several measures of statistical importance proved to be statistically significant for both IgG and IgM responses to A549 cells (Table 2), the single best value was the IgG response obtained by measuring the percentage of cells bound by IgG (p=0.0001), followed closely by the percentage of cells covered by IgM autoantibodies (p=0.0012).

By measuring the percentage of A549 cells bound by serum IgG and using a cutoff value of 88%, we were able to discriminate lung cancer patients with a sensitivity of 0.81 and a specificity of 0.80. Using a cutoff value of 85%, we were able to discriminate lung cancer patients with a sensitivity of 0.87 and a specificity of 0.6 (Table 3).

- Although we were able to obtain statistically significant results, we intend on obtaining more normal human serum controls to further elucidate the potential effectiveness of our screening method.
- Western blots reveal several proteins upregulated in the cancer cell lines and either repressed or not expressed in the immortalized normal cells! → vaccination!



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3. Pedchenko, T. "Early Detection of NSCLC with ScFv Selected against IgM Autoantibody." *PLoS One* 8.4 (2013).

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High-risk population in idiopathic pancreatic adenocarcinoma: guidelines for screening

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Introduction

Background

•Pancreatic cancer (PC) is one of the deadliest forms of cancer. It is projected to be the 10th most common cancer in the US in 2013, yet it will be the 4th leading cause of cancer death[1].
•PC confers an annual incidence to death ratio of 0.92 because more than 95% of PC diagnosis is Stage 2 or greater, chemotherapy is marginally active, and surgical therapy is extensive.
•Currently, the medical community does nothing to attempt early detection of PC, and little progress has been made in increasing PC survival rates.
•This leaves more than 40,000 people annually with a near 0% chance of survival[1].

Study objective

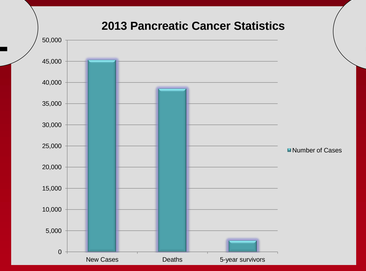
•Resection at an early stage remains the only hope for long-term survival.
•A screening program to detect PC at a resectable stage is needed. However, with a low incidence rate of about 1%, a screening program would be beneficial only for high-risk individuals (HRIs)[2].
•Many risk factors for PC have been suggested and quantified, but no true high-risk population has been definitively identified for a screening program. This is particularly true for idiopathic/sporadic pancreatic adenocarcinoma patients, which represent 90-95% of PC sufferers.
•This study asserts that a true higher risk population does exist in idiopathic pancreatic adenocarcinoma, and proposes simple guidelines for screening.

Methods

- A systematic review was conducted of the literature regarding identification of and screening in high-risk groups.

Results

1



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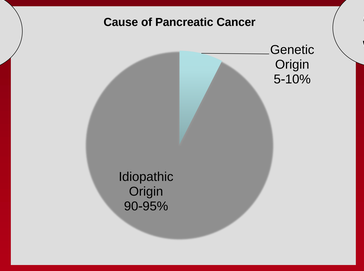

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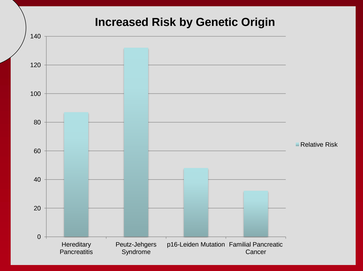

1 – 2013 Epidemiology Statistics for Pancreatic Cancer[1]

2 – Causes of pancreatic cancer: genetic origin vs. idiopathic origin[3]

3 – Increased risk for PC, conferred by genetic risk factors (relative risk compared to general population)[4,5,6,7]

4

New-Onset Diabetes and Advanced Age		
Study	**Study Population**	**Findings (95% CI)**
Chari, et al. 2005	New-Onset DM >50 yrs old	RR = 7.94 (4.70-12.55)
	New-Onset DM 50-70 yrs old	RR = 5.23 (1.70–12.20)
	New-Onset DM ≥70 yrs old	RR = 9.91 (5.26–16.96)
Hart, et al. 2011	New-Onset DM with PC	Mean age at PC dx = 76.4 ± 6.8
	New-Onset DM without PC	Mean age at index date = 71.7 ± 9.7
Lee, et al. 2012	New-onset DM with PC	Mean age at PC dx = 61.3 ± 10.2
	New-onset DM without PC	Mean age at index date = 55.8 ± 10.3
Aggarwal, et al. 2012	New-onset DM with PC	Median age at PC dx = 76 (range 39-90)
Mizuno, et al. 2013	New-onset DM ≥55 yrs old	OR*, per year over age 55 = 1.12 (1.03-1.24)
	Subjects with new-onset DM, <55 yrs old, with PC	0/19 patients
	Subjects with new-onset DM, ≥55 yrs old, with PC	7/21 patients

*Multivariate Analysis
DM diabetes mellitus, PC pancreatic cancer, CI confidence interval, dx diagnosis, RR risk ratio, OR odds ratio

5

New-Onset Diabetes and Weight Loss		
Study	**Study Population**	**Mean weight change (kg ± standard deviation)**
Hart, et al. 2011	New-Onset DM with PC	At DM dx = (-2.1 ± 3.8)
	New-Onset DM without PC	At DM dx = +1.4 ± 4.7
	New-Onset DM with PC	At PC dx = (-8.3 ± 8.3)
	New-Onset DM without PC	At PC dx = (-0.8 ± 4.8)
Lee, et al. 2012	New-Onset DM with PC	(-4.1 ± 4.7)
	New-Onset DM without PC	(-0.4 ± 1.5)
Aggarwal, et al. 2012	New-Onset DM with PC	At DM dx = (-2.2) (range [(-36)-7.4])*
	Longstanding DM with PC	At DM dx = +2.5 (range [(-2)-16])*

*Median weight change

DM diabetes mellitus, PC pancreatic cancer, dx diagnosis

6

Risk Factor + Smoking		
Study	**Study Population**	**Findings (95% CI)**
Hereditary Pancreatitis + Smoking		
Lowenfels, et al. 2001	HP with smoking vs. HP without smoking	AOR^ = 2.1 (0.7-6.1)
CDKN2A + Smoking		
McWilliams, et al.	*CDKN2A* mutation + current smoker	HR = 25.8
Familial Pancreatic Cancer + Smoking		
Klein, et al 2004	FPC kindred with at least 1 FDR with PC + Ever smoker	SIR = 19.2 (7.7–39.5)
	FPC kindred with at least 1 FDR with PC + Never smoker	SIR = 6.25 (1.70–16.0)
New-Onset Diabetes + Smoking		
Chari, et al. 2005	New-Onset DM + Smoking	OR^ = 5.84 (0.62–55.43)
Ben, et al.	New-Onset DM + Smoking	AOR^ = 4.77 (2.71–8.38)
	New-Onset DM without Smoking	AOR^ = 4.39 (3.28–5.89)

^AOR Age- and sex- adjusted odds ratio
^Conditional Logistic Regression Analysis
*Logistic Regression Analysis, AOR odds ratio adjusted for age, history of diabetes, family history of pancreatic cancer, heavy alcohol consumption and smoking status.
DM diabetes mellitus, PC pancreatic cancer, CI confidence interval, dx diagnosis, HP Hereditary Pancreatitis, SIR standard incidence ratio, FPC familial pancreatic cancer, FDR first-degree relative

4 – Increased pancreatic cancer risk in new-onset diabetes with advanced age [8,9,10,11,12]

5 – Increased pancreatic cancer risk in new-onset diabetes with weight loss [9,10,11]

6 – Increased pancreatic cancer risk with any risk factor + smoking [7,13,14,15]

Conclusions

The definition of a high-risk population for PC should include

•Those with a genetic predisposition – hereditary pancreatitis or PJS, carriers of *p16-Leiden* mutations, and FPC patients with a ≥7-fold risk determined by PancPro.
•Those with a clinical predisposition (idiopathic PC) – new-onset diabetes over the age of 50 and a history of smoking. Weight loss in this group should be an additional risk consideration.
•This definition of HRIs will encompass those at the greatest risk for PC, including those with idiopathic PC – a group that has largely been ignored in screening discussions but that is in the greatest need of recognition.

Guidelines for screening in high-risk groups

•While knowledge regarding PC is far from complete, we have reached a time when standing by should no longer be an option.
•These groups see an elevated risk of at least 7-fold and up to 132-fold. When compared to the levels of risk that warrant screening for other types of cancer, screening in these high-risk groups should certainly be warranted, as well.
•A screening protocol must be officially established, accepted, and put into practice for these groups, in order to use what we do know to help those who will fall prey to this deadly disease.
•The first step of screening should fall on the primary care physician to recognize those at high risk.

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Role of p38 in AS1411 Activity

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Abstract

The anticancer agent, AS1411, is a G-rich phosphodiester oligodeoxynucleotide, which forms a stable quadruplex structure and binds specifically to nucleolin as an aptamer. It efficiently inhibits proliferation and induces cell death in many types of cancer cells, but has little effect on normal cells. We have also shown that AS1411 is taken up by macropinocytosis and stimulates further macropinocytosis by a nucleolin-dependent mechanism in several cancer cells. AS1411 activity correlates with stimulated macropinocytosis, suggesting this hyperstimulation of macropinocytosis may explain the unusual cancer cell death caused by AS1411. Macropinocytosis is a ligand-independent endocytic pathway that is normally activated by growth factor receptor stimulation. One of the downstream effectors of phosphorylated EGFR, p38, has been shown to become activated when treated with AS1411. Therefore in this study, we investigated the participation of the EGFR signaling pathway, specifically the role of p38, in AS1411-induced macropinocytosis and cell death. Pre-incubation of DU145 cells with a specific p38 siRNA did not significantly alter the survival of cell lines treated with AS1411. Furthermore, pre-incubation with the same p38 siRNA did not significantly inhibit the stimulation of AS1411-mediated macropinocytosis. We also found that following pre-incubation of DU145 cells with a specific EGFR inhibitor, the AS1411-dependent interaction between Nonmuscle myosin IIA (NMIIA) and EGFR is inhibited. These results suggest that the activation of p38 is not critically involved in the effect of AS1411 on cancer cells.
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Background

- AS1411 induces macropinocytosis (MP) in cancer cells, but not in non-malignant cells, providing a possible explanation for its cancer selectivity
- MP is a form endocytosis that requires reorganization of the actin cytoskeleton to form large vesicles called macropinosomes
- MP occurs in cancer cells in response to growth factor receptor activation (e.g. EGFR) via activation of downstream effectors such as Ras, PI3K, and Rac1, and p38
- p38, a stress induced MAPK, phosphorylates EGFR and promotes its internalization into vesicles
- p38 is activated in cells treated with AS1411
- AS1411, when bound to its target, nucleolin, can result in activation of EGFR
- Induction of MP by AS1411 requires nucleolin
- AS1411 causes nonapoptotic cancer cell death, similar to that induced by hyperstimulation of MP

Objectives

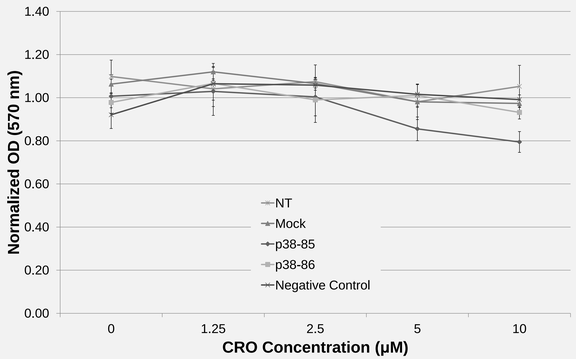
- To investigate the role of p38 in the induction of AS1411 mediated cancer cell death via nucleon-dependent MP.
- Explore for other possible mechanisms of how AS1411 induces nucleolin-dependent MP

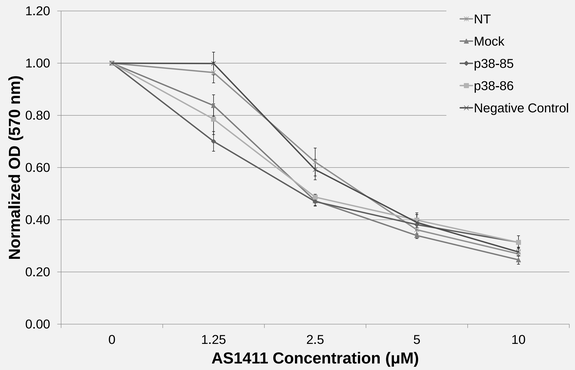
Results

Figure 1

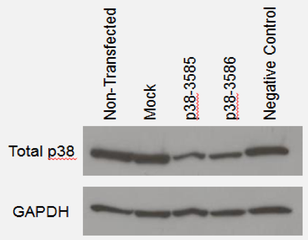
AS1411 Antiproliferative Activity Does Not Depend on p38 Activity

A.





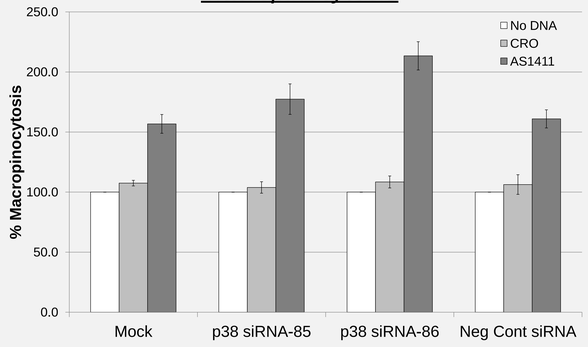
B.



A. Transfected DU145 cells were plated at low density and incubated 18 hours at 37°C to allow adherence. They were treated with different concentrations of CRO (upper panel) and AS1411 (lower panel). After 5 days of treatment, cell proliferation was determined by MTT assay.
B. Total p38 expression of the transfected DU145 cells (upper panel) was analyzed by Western Blot analysis. GAPDH was used as a loading control (lower panel).

Figure 2

p38 is Not Required for AS1411-Induced Macropinocytosis



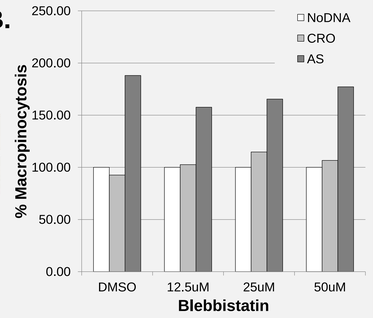
Transfected DU145 cells were plated at low density and incubated 18 hours at 37°C to allow adherence. They were treated with vehicle (No DNA), CRO, or AS1411 at a final concentration of 10 μM at 37°C for 48 hrs. After treatment, dextran 10 kDa-Alexa488 (macropinocytosis marker) was added, incubated 30 minutes at 37°C and washed. Cells were then processed to be analyzed by flow cytometry.

Figure 3

AS1411 Activity May be Dependent on NMIIA

A.

B.



A. DU145 cells were untreated (-) or treated with either CRO (C) or AS1411 (AS) at a final concentration of 10 μM at 37°C for 24 or 48 hrs. After incubation, the cells were lysed and EGFR was immunoprecipitated from the cell lysates and analyzed by immunoblotting with anti-NMIIA (upper panel). GAPDH was used as a loading control (lower panel)
B. DU145 cells were incubated with vehicle (No DNA), CRO, or AS1411 at a final concentration of 10 μM at 37°C for 48 hrs. After incubation, cells were treated by adding serum free medium with varying concentrations of Blebbistatin for 1 hr at 37°C. After treatment, dextran 10 kDa-Alexa488 was added, incubated 30 minutes at 37°C, and washed. Cells were then processed to be analyzed by flow cytometry

Conclusions

- p38, although activated in response to treatment of cells with AS1411, is not essential to the antiproliferative activity of AS1411.
- p38 also does not play a role in the induction of macropinocytosis
- p38 may play more of a protective role in preventing overactivation of macropinocytosis.
- The activation of the p38 in response to AS1411 treatment is likely just the cells standard response to stress.
- Nonmuscle myosin IIA might play an important role in the activity of AS1411 in cancer cell lines.
- Interaction between nonmuscle myosin IIA and EGFR, upregulated in cells treated with AS1411, might be involved in the antiproliferative activity of AS1411 on cancer cell lines.
- Nonmuscle myosin IIA activity might also be important for the activation of macropinoctosis by AS1411.

Future Plans

- Determine the role of nonmuscle myosin IIA in AS1411-induced macropinocytosis and cell death.
- Identify alternative molecular mechanisms that regulate AS1411 effects on signaling and trafficking.

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